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Detection of Active Butyrate-Degrading Microorganisms in Methanogenic Sludges by RNA-Based Stable Isotope Probing▽†

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Butyrate-degrading bacteria in four methanogenic sludges were studied by RNA-based stable isotope probing. Bacterial populations in the ^{13}C-labeled rRNA fractions were distinct from unlabeled fractions, and *Syntrophaceae* species, *Tepidanaerobacter* sp., and *Clostridium* spp. dominated. These results suggest that diverse microbes were active in butyrate degradation under methanogenic conditions.

Butyrate is one of the important intermediates in the degradation of organic matter under methanogenic conditions (14, 16). Under these conditions, butyrate degradation is carried out by a syntrophic association of butyrate-oxidizing bacteria and hydrogenotrophic methanogens, because of thermodynamic constraints (17). Due to the fastidious nature of this syntrophic metabolism, isolation of butyrate-degrading syntrophs has been difficult and thus information on butyrate-degrading bacteria is based on some isolates belonging to the family *Syntrophomonadaceae*. Due to this lack of knowledge and the lack of appropriate molecular markers, culture-independent studies have focused only on species of the family *Syntrophomonadaceae* (5, 15, 25). Consequently, the natural diversity of syntrophic butyrate-degrading bacteria has not been studied in any detail.

The recent development of stable isotope probing (SIP) enables metabolic function and taxonomic identity to be examined concurrently (3). Only one study on methanogenic butyrate degradation has been reported with this technique (1), but not within waste/wastewater-treated methanogenic sludges. SIP provides a potentially fruitful tool for identifying potential butyrate-degraders in a methanogenic environment. In this study, therefore we used RNA-based SIP (RNA-SIP) with [$^{13}C_4$]butyrate as a substrate to explore the microorganisms involved in butyrate degradation in four methanogenic sludges.

Four methanogenic sludges were used in this study. Mesophilic granular sludge MP and thermophilic granular sludge TP were taken from two lab-scale multistage upflow anaerobic sludge blanket reactors treating palm oil mill effluent. Mesophilic anaerobic digester sludge treating palm oil mill effluent (sludge MBF) and thermophilic digester sludge treating municipal solid waste (sludge JET) were taken from commercial plants. Detailed properties of these sludges were described in our previous report (7). Incubation was carried out anaerobically at 37°C (for mesophilic sludges) or 55°C (for thermophilic sludges). The granular sludges TP and MP were preincubated with 5 mM butyrate because of prolonged storage at 4°C for over 2 years. Degradation of butyrate was monitored by measuring methane production using gas chromatography as described previously (6). Preincubation was conducted for 14 days, added butyrate was completely converted to methane, and then the sludge was sampled as an unlabeled control microbial consortium. Digester sludges MBF and JET were used immediately after sampling, and RNA extracted from unincubated sludges was used as an unlabeled control RNA. Incubation of stable isotope-labeled substrate was performed by a previously described method (7), using [$^{13}C_4$]butyrate (Isotec, Miamisburg, OH) at a concentration of 5 mM (0.12 mmol). During incubation, over 95% of [$^{13}C_4$]butyrate was converted to methane in 3 and 7 days for sludges TP and MP, respectively. For sludges MBF and JET, [$^{13}C_4$]butyrate was mostly (over 80%) converted to methane within 5 and 2 days, respectively. After the incubation, the sludge (~5 ml) was sampled. This [$^{13}C_4$]butyrate incubation step was repeated to obtain the second incubated sludge. The incubation experiment was repeated twice at almost the same methanogenesis rate.

Total RNA extraction from the collected sludge samples and RNA purification were conducted by the method described previously (21). RNA was separated by equilibrium density gradient centrifugation and fractionated. The bacterial rRNA content in each fraction was quantified by quantitative reverse transcription-PCR (RT-PCR) with bacterial universal primers (6). The unlabeled control rRNA gradient showed peaks at a buoyant density (BD) around 1.77 g · ml^{-1}, which is characteristic of unlabeled bacterial rRNAs in cesium trifluoroacetate (see Fig. S1 in the supplemental material) (12). In sludge MP, the rRNA gradient profile for the first incubation sludge had an increase in heavier (>1.78 g · ml^{-1}) rRNA, but the peak BD was almost the same as in control rRNA (see Fig. S1A in the supplemental material). In sludges JET, TP, and

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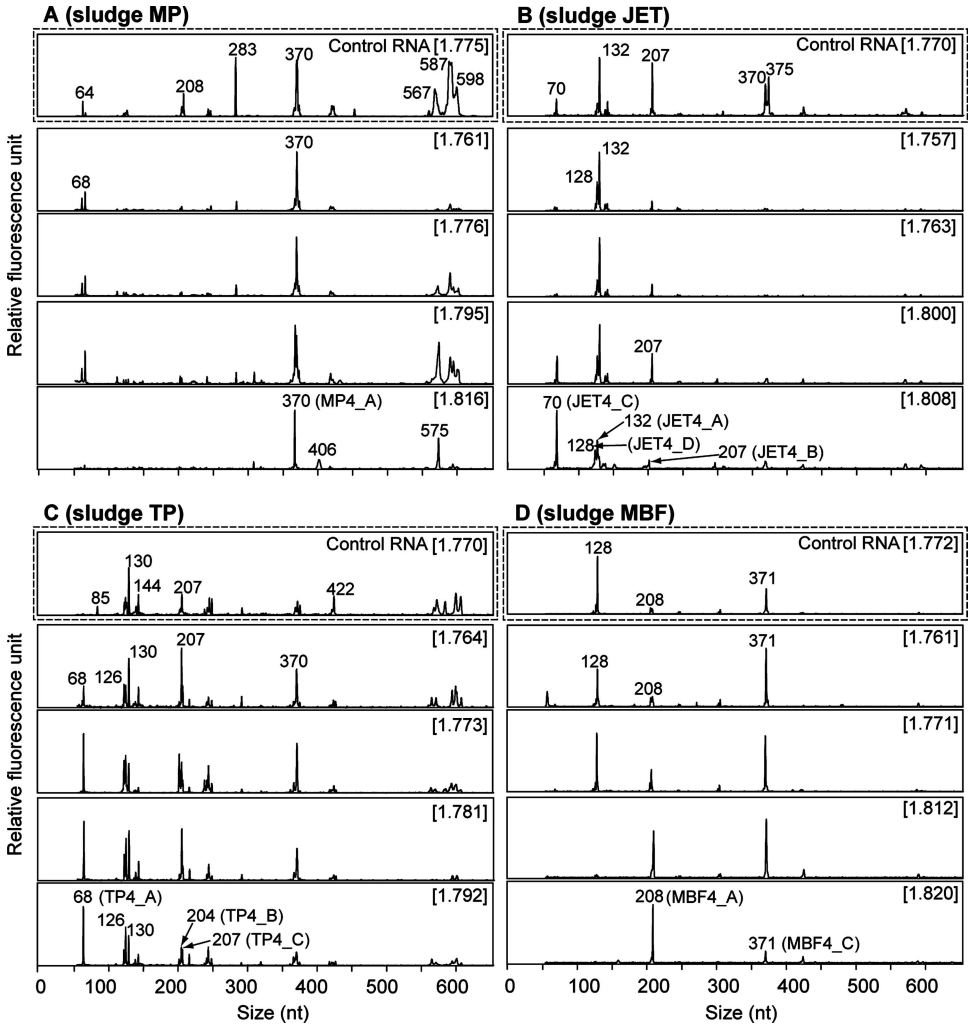

FIG. 1. Bacterial T-RFLP fingerprinting of density-resolved RNA from representative ^{13}C-labeled and unlabeled fractions of sludges MP (A), JET (B), TP (C), and MBF (D). Electropherograms were generated from gradient fractions of RNA from the first [$^{13}C_4$]butyrate incubation as a template. Dashed boxes show T-RFLP fingerprints of density-resolved unlabeled control RNA. Cesium trifluoroacetate BDs (g · ml^{-1}) of gradient fractions are given in brackets. The numbers at the T-RF peaks indicate the T-RF lengths. Representing phylotypes of T-RFs identified by clone analysis are indicated in parentheses.

MBF, after the first incubation with [$^{13}C_4$]butyrate, the rRNA profile gradually shifted toward the heavier BDs and a tailing of ^{13}C-enriched (>1.8 g · ml^{-1}) rRNA was detected (see Fig. S1B, C, and D in the supplemental material). After the second incubation, the entire rRNA BDs shifted toward the heavier fraction in all sludges (see Fig. S1 in the supplemental material). We used the RNAs extracted from the first [$^{13}C_4$]butyrate incubation sludges for further analysis, because all sludges could be effectively labeled by the first [$^{13}C_4$]butyrate incubation. This pulse sampling could avoid extended incubation and minimize carbon cross-feeding (13).

The representative bacterial terminal restriction fragment length polymorphism (T-RFLP) fingerprint profiles of ^{13}C-labeled and unlabeled fractions from [$^{13}C_4$]butyrate incubation sludges and control RNA are shown in Fig. 1. We constructed clone libraries from the heaviest RNA fractions and analyzed 50 clones for each library as described previously (6). The phylogenetic affiliations of all analyzed bacterial clones are summarized in Table 1, and the positions of selected phylotypes, represented by at least four clones, are shown in Fig. 2.

The 370-bp terminal restriction fragment (T-RF) was dominant throughout the fractions of sludge MP (Fig. 1A). The T-RF corresponds to phylotype MP4_A (39 clones), and the most closely related isolate was *Clostridium orbiscindens* (96% of 16S rRNA gene sequence similarity) (Fig. 2). The 575-bp T-RF also dominated the ^{13}C-labeled fraction (Fig. 1A) but was not detected in clones that indicate the 575-bp T-RF. Since we used different primer sets for T-RFLP analysis and construction of clone library, it might be influence these results. Within sludge JET, the 132-bp T-RF was dominant in fingerprints generated from unlabeled control RNA (Fig. 1B), but not after [$^{13}C_4$]butyrate incubation. The 70-bp T-RF increased in relative abundance, specifically in the ^{13}C-labeled fraction, corresponding to the second-most-abundant phylotype, JET4_C, belonging to *Clostridium* cluster IV (Fig. 2). Other dominant phylotypes JET4_A (12 clones) and JET4_D (6

TABLE 1. Phylogenetic affiliation and numbers of bacterial 16S rRNA clones retrieved from clone libraries generated from ^{13}C-labeled RNA fractions

Phylogenetic group[a]	No. of clones				T-RF length of clones (bp)[b]
	MP	JET	TP	MBF	
Bacteroidetes	1	9	5	6	**207**, **370**, 371
Clostridium	46	14		2	**68**, **70**, 369, **371**
Syntrophomonas				1	299
Syntrophothermus			2		372
Tepidanaerobacter		7	19		**68**
Desulfotomaculum		1	1		132, 244
Syntrophaceae				31	**208**
Clone MST group				5	**248**
Petrobacter	3				**313**
Synergistes			2		130
Spirochaetes				4	**129**
Coprothermobacter		12			**132**
Anaerobaculum		6	2		**128**
EM3			8		**204**
OP7			1		130
OP5			3		72, 132
OP10			3		**127**
Marine group A			3		**207**
WWE1				1	305
NKB19			1		210
Unidentified		1			73
Total	50	50	50	50	

[a] Phylogenetic groups with cultivated representatives are named according to the taxonomic outline of *Bergey's Manual of Systematic Bacteriology* (4). Candidate phyla are named based on the review by Hugenholtz (9).

[b] Phylotypes that are represented by at least three clones and whose sequence length matches the lengths of specific T-RFs are highlighted in boldface, and others had T-RF lengths predicted using sequence data. Note that measured T-RFs are typically 0 to 3 bases shorter than the predicted T-RFs (24).

clones) were closely related to *Coprothermobacter* spp. and *Anaerobaculum* spp., respectively, but intensities of corresponding T-RFs were reduced toward heavier fractions (Fig. 1B). Phylotype JET4_B (nine clones) was deeply branched within phylum *Bacteroidetes*, with T-RFs corresponding to 207 bp (Fig. 2). No known butyrate-oxidizing bacteria were sequenced from the sludges MP and JET. Based on these results, we speculate that *Clostridium* spp. of phylotypes MP4_A and JET4_C may be closely linked to the degradation of butyrate in sludges MP and JET, respectively (Fig. 2). Chauhan and Ogram also reported the *Clostridium* sequence, derived from their DNA-SIP study using butyrate, might be represent a novel butyrate-oxidizing bacterium (1).

Diverse T-RFs were detected from sludge TP, and the major T-RFs of 130, 207, and 370 bp in the unlabeled light fractions were markedly reduced in abundance in the ^{13}C-labeled fractions (Fig. 1C). The 68-bp T-RF that dominated the ^{13}C-labeled fraction (Fig. 1C), corresponding to the most abundant phylotype, TP4_A (19 clones), was closely related to *Tepidanaerobacter syntrophicus* and an anaerobic strain, TOL (Fig. 2). Strain TOL is likely involved in palmitate degradation, as previously indicated (6), and thus phylotype TP4_A might also be involved in butyrate degradation.

Within sludge MBF, phylotype MBF4_A (30 clones), representing a 208-bp T-RF, clustered with the family *Syntrophaseae* and was closely related to clone BLB04, with 99% 16S rRNA gene sequence similarity. Clone BLB04 was the most abundant

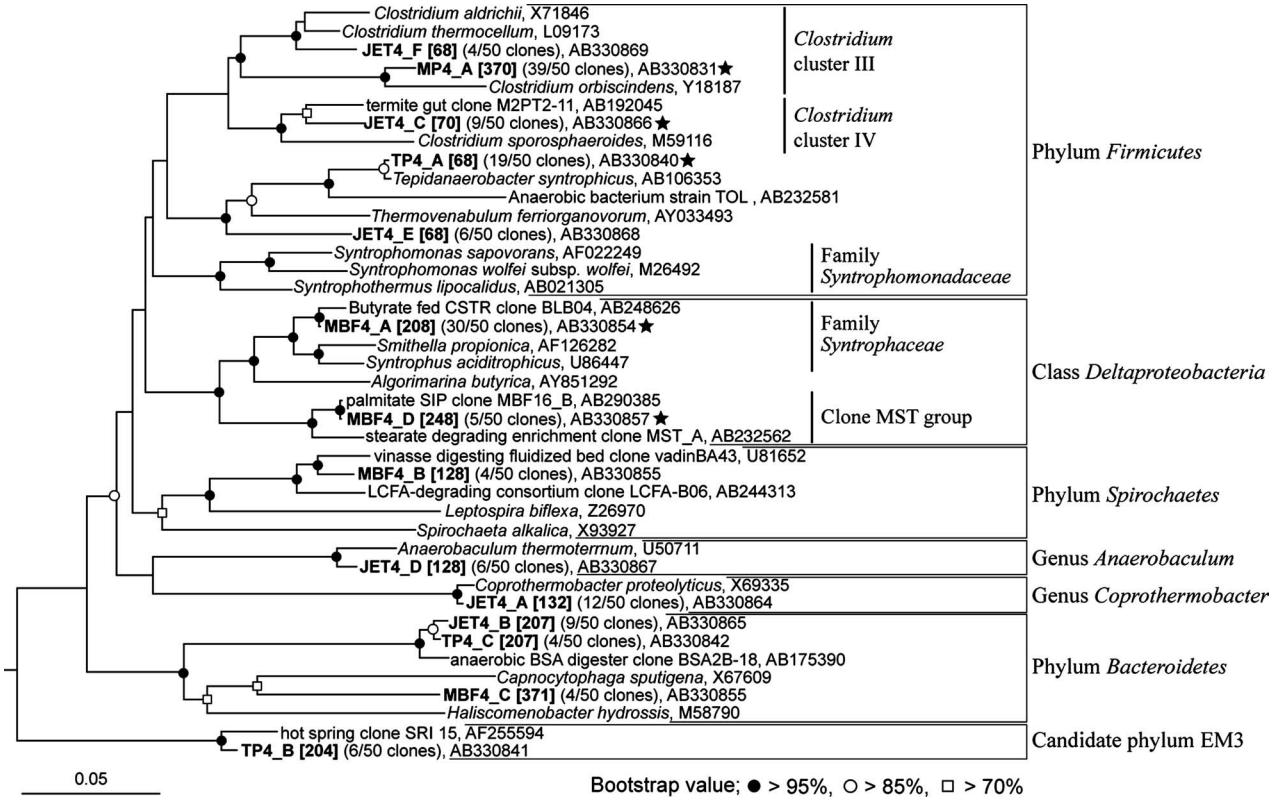

FIG. 2. Phylogenetic placement of representative bacterial 16S rRNA phylotypes from ^{13}C-labeled RNA fractions. The phylogenetic tree was constructed by the neighbor-joining method. The measured T-RF lengths of phylotypes digested with MspI are shown in brackets. The scale bar represents the number of changes of nucleotides per sequence position. The symbols at each branch point show the bootstrap values obtained with 1,000-resampling analysis. Stars indicate putative butyrate degraders inferred from our results.

clone retrieved from a butyrate-fed methanogenic chemostat at a low dilution rate (23), and cultured related species of *Smithella propionica* and *Syntrophus aciditrophicus* were known to degrade butyrate (10, 11). The second-most-abundant phylotype was MBF4_D (five clones), but its corresponding T-RF of phylotype MBF4_D of 248 bp was not detected (Fig. 1D). Phylotype MBF4_D was clustered with the clone MST group of the class *Deltaproteobacteria*, which might be considered a novel fatty acid-degrading bacterial group (6, 7). Thus, based on our results and previous findings (6, 7, 10, 11, 23), microbes detected as phylotypes MBF4_A and MBF4_D likely degrade butyrate in sludge MBF.

The community analysis of ^{13}C-labeled RNA showed that different species were retrieved from different sludges. We have detected only three clones (two clones from sludge TP and one clone from sludge MBF) related to *Syntrophomonadaceae* species that were typically shown to be fatty acid-degrading microorganisms under the methanogenic conditions in this study (Table 1). Previously, Zellner et al. reported that organisms related to *Syntrophomonadaceae* species were not abundant enough to contribute to the observed butyrate turnover rate in the bioreactors (25). They concluded that the main syntrophic butyrate-oxidizing bacteria in the reactors might be an as-yet-unknown species. Recently, Tang et al. also showed that non-*Syntrophomonadaceae* species were dominant in a butyrate-fed methanogenic chemostat (23). Given the previous reports and our results, non-*Syntrophomonas* bacteria may also have a role in butyrate degradation in methanogenic environments. However, our result might be affected by longer periods of sludge storage, especially for sludge MP, resulting in survival of spore-forming bacteria. Furthermore, we need unlabeled substrate control experiments for comments on the impact of community change upon incubation, which is a potential limitation of this study.

During the β-oxidation of butyrate, cross-feeding of intermediately formed [^{13}C]acetate could label the non-butyrate degrader. Some clostridial species could degrade acetate in syntrophic association with hydrogenotrophic methanogens (8, 19), and *Syntrophus* spp. have also been reported to utilize acetate (2). However, acetate is converted to methane by aceticlastic methanogens in methanogenic sludges immediately (22) and [^{13}C]acetate might be assimilated by methanogenic archaea, because archaeal rRNAs also became heavier (see Fig. S1 in the supplemental material). Furthermore, syntrophic acetate oxidation tends to occur under certain conditions, such as high ammonium or volatile fatty acid concentrations (18, 20). Therefore, the dominant sequences identified here were considered to be labeled as a result of utilization of [$^{13}C_4$]butyrate. Application of SIP with [^{13}C]acetate could elucidate the flow of acetate in the methanogenic sludges.

Nucleotide sequence accession numbers. The 16S rRNA gene sequence data obtained in this study have been deposited in the GenBank/EMBL/DDBJ database under accession no. AB330831 to AB330871.

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